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14. ABSTRACT: BCL-2 family proteins regulate apoptosis but their mechanisms of action remain unclear. We have demonstrated that Bax and Bak, two pro-apoptotic members of the family, are both capable of triggering release of Ca ²⁺ from the endoplasmic reticulum and that this contributes to cell death in human prostate cancer cells. In work supported by this proposal we performed comprehensive analyses of the effects of siRNA-mediated knockdown of BH3-only members of the family, Bax, and Bak on apoptosis induced by diverse stimuli. The data demonstrate that Bax and Bak play non-overlapping roles in cell death; Bax appears to be linked more closely to cell death induced by stimuli that mobilize ER Ca ²⁺ , whereas Bak appears to play a more dominant role in ER stress. In ongoing studies we are assessing the effects of knockdown on ER Ca ²⁺ fluxes and plan to finish the project within a year.					
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INTRODUCTION

B cell lymphoma-leukemia-2 (BCL-2) was cloned in the mid-1980's at the t(14;18) chromosomal translocation that is the hallmark feature of non-Hodgkin's follicular B cell lymphoma (1, 2). Subsequent studies demonstrated that BCL-2 is a member of a larger, evolutionarily-conserved gene family that includes proteins with pro- and anti-apoptotic properties (3). Although the family plays a critical role in regulating a central point in the apoptotic pathway (release of pro-apoptotic proteins from mitochondria), precisely how it does so remains a matter of intense investigation. Pro-apoptotic members of the family can form transmembrane pores in the outer mitochondrial membrane, and anti-apoptotic members of the family can block these effects (4, 5). However, the proteins can also localize to the endoplasmic reticulum and nuclear envelope, and whether or not functions at these other sites play important roles in cell death regulation remains unclear.

Although BCL-2 family proteins play very broad roles in regulating apoptosis, they appear to be particularly relevant in prostate cancer. Early work demonstrated that BCL-2 expression increases during prostate cancer progression, particularly when tumors become androgen-independent (6). Enforced overexpression of BCL-2 in androgen-sensitive LNCaP cells is sufficient to render the cells androgen-independent (7), and we showed that the process of selecting metastatic variants of LNCaP resulted in the isolation of cells that overexpressed BCL-2, were androgen-independent, and were resistant to chemotherapy-induced apoptosis (8). A small molecule inhibitor of BCL-2 (ABT-737) has been developed by Abbott Laboratories (9), and there is great enthusiasm for testing its anti-tumor activity in patients with androgen-independent prostate cancer.

In work leading up to this proposal we showed that two pro-apoptotic members of the BCL-2 family (Bax and Bak) can localize to the endoplasmic reticulum (ER) and trigger release of ER-sequestered Ca^{2+} , resulting in mitochondrial Ca^{2+} uptake and cytochrome c release (10, 11). However, the precise biochemical mechanisms involved in these effects were not elucidated. Our overall goal was to identify these mechanisms, focusing on possible direct effects of Bax and Bak on the ER membrane. However, parallel technical and scientific advances revealed a new layer of complexity with regard to the consequences of ER Ca^{2+} release (the induction of ER "stress"), and our results indicate that the roles of Bax and Bak in responding to ER stress are distinct.

BODY

Statement of Work

- 1. Define the effects of mitochondrial Ca^{2+} uptake on cytochrome c mobilization and release.** Since the association between cytochrome c and the mitochondrial inner membrane is sensitive to cardiolipin charge and redox status, we speculate that the function of mitochondrial Ca^{2+} uptake during apoptosis is to promote cytochrome c mobilization. We will measure cytochrome c mobilization in isolated mitochondria exposed to various concentrations of Ca^{2+} and in whole cells exposed to Ca^{2+} -dependent and -independent proapoptotic stimuli in the absence or presence of inhibitors of mitochondrial Ca^{2+} uptake.
- 2. Determine the effects of BH3-only members of the BCL-2 family on intracellular Ca^{2+} fluxes.** We speculate tBid preferentially catalyzes Ca^{2+} -independent cytochrome c release, whereas other BH3-only proteins trigger Bax- and Ca^{2+} -dependent cytochrome c release via disruption of the ER Ca^{2+} pool. We will directly test this possibility by monitoring the

movements of BH3 proteins in whole cells exposed to a panel of different proapoptotic stimuli. We will also compare the effects of exogenous native and ER-targeted forms of tBid and other BH3-only proteins (Bim, Bik) on Bax/Bak oligomerization, depletion of ER Ca^{2+} , and cell death by transient transfection and adenoviral gene transfer.

3. Identify the direct effects of Bax and Bak on ER Ca^{2+} fluxes. We will use ER-targeted forms of Bax and Bak to investigate whether or not they empty the ER Ca^{2+} pool via direct mechanisms. Effects of exogenous Bax/Bak alone or in combination with BH3 proteins will be compared. Cytochrome c release will be measured in cells that are deficient in endogenous Bax or Bak reconstituted with native or mitochondrial forms of the proteins to define the involvement of mitochondrial outer membrane effects in cytochrome c mobilization versus release. We will also test the effects of exogenous Bax and Bak (with or without BH3 proteins) on ER Ca^{2+} uptake in isolated microsomes.

PROGRESS

We prioritized the whole cell experiments described in Aim 2 over the isolated organelle experiments described in Aims 1 and 3 because we felt that they would have greater biological relevance. We do intend to complete the experiments described in all 3 Specific Aims and submit the work for publication within the next year. In addition, observations we made in the course of performing the experiments described in Aim 2 serve as the foundations for new projects on ER stress that have high, immediate translational potential.

We proposed to overexpress wild-type or targeted forms of various BCL-2 family proteins in human prostate cancer cells to determine whether or not they regulate apoptosis via effects on intracellular Ca^{2+} compartmentalization. David Andrews pioneered this approach (12, 13), and David provided us with his constructs. However, as outlined in the progress report for Year 1, we discovered that cells that were “stably” transfected with the constructs lost protein expression rapidly, typically within 2 weeks after we had isolated clones. Furthermore, it appeared that overexpression of mitochondrially-targeted forms of the proteins was toxic, consistent with Distelhorst’s findings in human breast cancer cells (14).

The commercialization of siRNA technology allowed us to overcome this pitfall by adopting one of the alternative approaches we outlined in the original application. Rather than overexpress specific BCL-2 proteins, we used siRNA to transiently knock down expression of several proapoptotic BCL-2 family members (Bax, Bak, Bid, Bim, Puma and Noxa) and examine how loss-of-function affected apoptosis induced by a panel of relevant stimuli. Most investigators feel that protein knockdown is a better means of determining function than overexpression because an overexpressed protein can adopt functions that are not relevant to the effects it has when it is expressed at physiological levels. Following is a summary of our findings.

Roles of BH3-only proteins on apoptosis. Before characterizing the effects of the BH3-only proteins on Ca^{2+} fluxes, we used siRNA-mediated gene silencing to determine their overall contributions to cell death in prostate cancer cells exposed to a variety of different pro-apoptotic stimuli. We first focused attention on Bid and Bim because they are considered direct activators of Bax and Bak, whereas the other BH3-only proteins appear to act more indirectly (as “sensitizers”) (15, 16). In every experiment we confirmed that silencing was efficient by immunoblotting and discounted any experiment in which silencing efficiency was less than 50% (see **Figure 1** for typical results, in this case in LNCaP-Pro5 cells). Overall, our results demonstrated that Bid and Bim both contributed to cell death induced by docetaxel (taxotere), bortezomib (PS-341), staurosporine, and thapsigargin (**Figure 2 and data not shown**).



Figure 1: Representative example of silencing efficiency in LNCaP-Pro5 cells. Cells were transiently transfected with siRNAs targeting Bid or Bim (or non-targeting siRNAs) for 48 h, and protein expression was determined by immunoblotting. Blots were then stripped and reprobed for actin (loading control). Densitometric quantification of silencing efficiency demonstrated that knockdown averaged about 80% in LNCaP-Pro5, PC-3, and DU-145 cells.

Time course studies demonstrated that siRNA-mediated knockdown of Bim inhibited staurosporine-induced apoptosis fairly well at early time points as measured by PI/FACS and detection of active caspase-3 by immunoblotting (**Figures 3,4**). Staurosporine is unique among our pro-apoptotic stimuli because it induces apoptosis with such rapid kinetics; similar analyses with the other agents demonstrated that exposure times of at least 24 h were required to observe measureable increases in DNA fragmentation or caspase-3 activation (data not shown). Staurosporine was one of the agents that induced endoplasmic reticular Ca^{2+} release in our previous studies (10, 11)(Progress Report, Year 2). Therefore, we are focusing on the role of Bim in staurosporine-induced Ca^{2+} fluxes in our ongoing studies.

Noxa and Puma are BH3-only proteins that have been implicated in DNA damage-induced, p53-mediated apoptosis (17-19). As part of our screen we investigated whether or not a panel of pro-apoptotic stimuli induced Puma or Noxa expression in LNCaP and/or PC-3 cells. The only agent that produced striking changes in expression of either protein was the proteasome inhibitor, bortezomib, which stimulated a rapid, concentration-dependent increase in Noxa expression in both cell lines (**Figure 5** and data not shown). Because proteasome inhibitors increase the half-lives of short-lived proteins, we suspect that protein stabilization contributes to the rapid Noxa accumulation observed in the cells, but we are also investigating the effects of

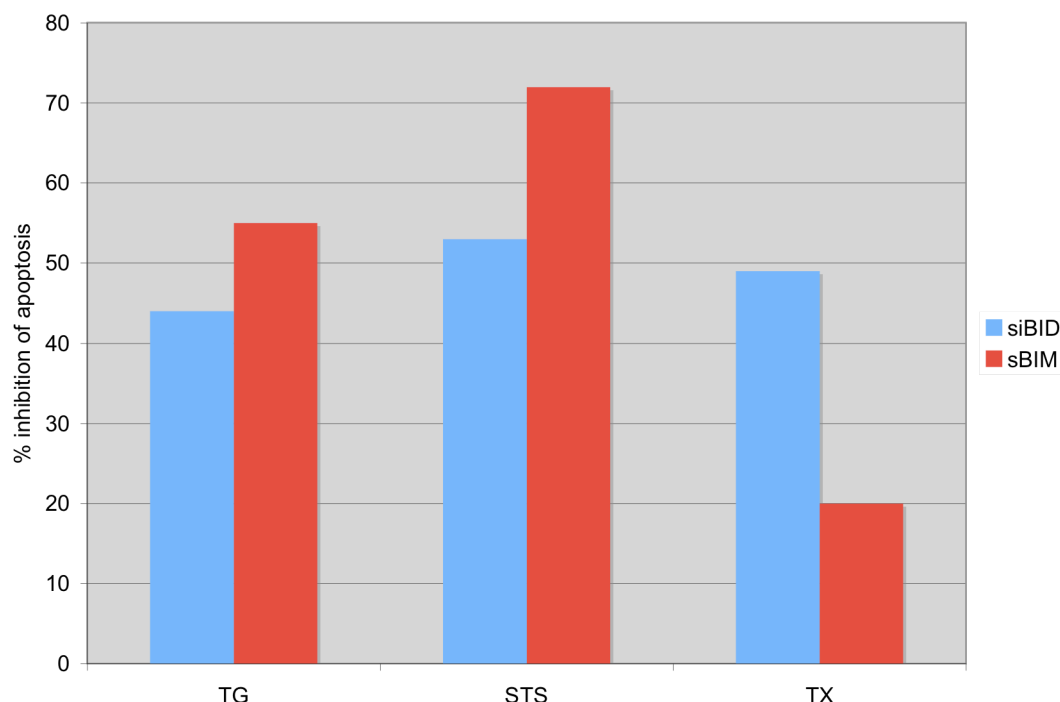


Figure 2: Effects of siRNA-mediated knockdown of “activator” BH3-only proteins on apoptosis. LNCaP-Pro5 cells were transiently transfected with siRNAs targeting Bid, Bim, or an off-target control for 48 h. Cells were then exposed to 1 μ M thapsigargin (TG), 500 nM staurosporine (STS), or 10 nM docetaxel (TX) for 48 h and DNA fragmentation was measured by PI/FACS. Mean percentage inhibition is displayed. Results are derived from at least 3 independent experiments.

the drug on Noxa mRNA levels by quantitative real-time PCR. We are currently trying to knock down Noxa to determine whether or not it is required for bortezomib-induced cell death; preliminary results suggest that it is. Importantly, Noxa appears to function as a selective inhibitor of the MCL-1 protein, which is the only major anti-apoptotic BCL-2 family member that is not inhibited by Abbott’s small molecule antagonist, ABT-737 (20, 21). Therefore, these observations have prompted us to examine the effects of the combination of ABT-737 plus bortezomib in our prostate cancer cells. Our data are also consistent with results obtained in melanoma cells (22, 23).

Roles of Bax and Bak in apoptosis. Our previous work demonstrated that overexpression of either Bax or Bak triggered ER Ca^{2+} efflux and mitochondrial Ca^{2+} uptake (11). Furthermore, previous studies employing mouse embryonic fibroblasts (MEFs) demonstrated that the proteins are largely redundant and that either is capable of mediating the proapoptotic effects of diverse agents (24). To more directly assess the contributions of each protein to cell death induced by diverse stimuli, we silenced Bax, Bak, or both proteins in LNCaP, PC-3, and DU-145 cells and measured the effects of knockdown on cell death. Immunoblotting confirmed that silencing suppressed expression of one or both proteins by over 80% (**Figure 6**). Knockdown of Bax alone or Bax plus Bak led to roughly equivalent effects on cell death induced by staurosporine or docetaxel (**Figure 7**). In contrast, silencing Bax had minimal effect on cell death induced by thapsigargin, whereas Bak silencing inhibited cell death as well as combined silencing of both Bax and Bak (**Figure 8**). Very similar effects were observed in cells exposed to bortezomib, where Bak also appeared to play a more important role than Bax in cell death induction (**Figure 9**).

Effects of proteasome inhibitors or thapsigargin on ER stress. We have previously used thapsigargin as a tool to induce ER Ca^{2+} release, mitochondrial Ca^{2+} uptake, and Ca^{2+} -dependent apoptosis. However, in addition to these effects on Ca^{2+} fluxes, thapsigargin can

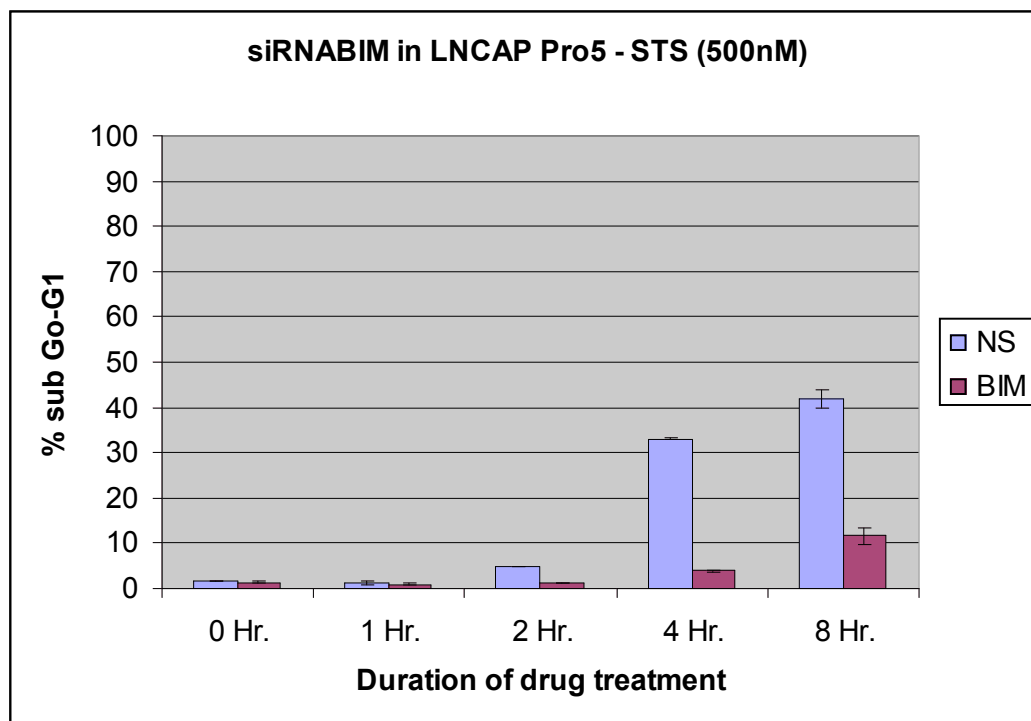
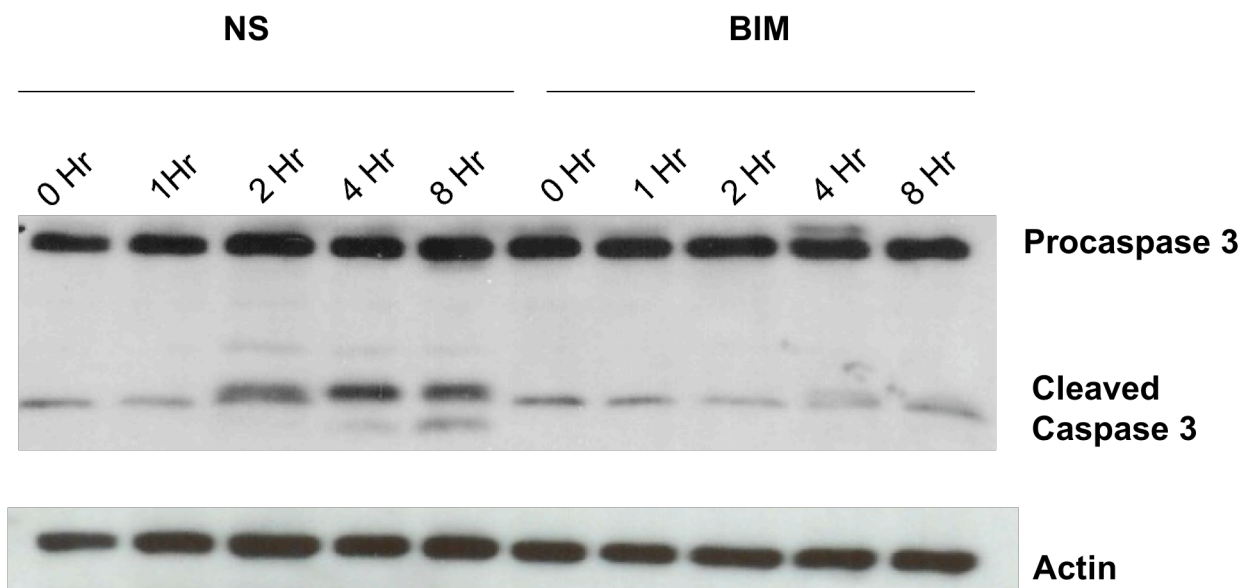


Figure 3 (Top): Effects of Bim silencing on caspase-3 activation. The presence of cleaved (active) caspase-3 was measured by immunoblotting. **Figure 4** (bottom): Effects of Bim silencing on apoptosis. Mean \pm SD, n = 3.

also regulate apoptosis indirectly by causing “endoplasmic reticular stress” (25). Furthermore, our recent work has demonstrated that ER stress is involved in proteasome inhibitor-induced apoptosis in human pancreatic cancer cell lines (26). Our finding that thapsigargin and proteasome inhibitors shared a unique dependency upon Bak for execution of cell death prompted us to compare their effects on ER stress in human prostate cancer cells. Bortezomib, another clinically relevant proteasome inhibitor (NPI-0052), and thapsigargin all induced several

features of ER stress (27), including phosphorylation of the translation initiation factor eIF2 α , suppression of protein synthesis, activation of the ER-resident caspase, caspase-4, and expression of ER stress-associated transcription factors (GADD153/CHOP and ATF-4) in LNCaP and PC-3 cells (**Figure 10** and data not shown). Therefore, we consider it likely that the Bak dependency observed may be related to Bak's role in mediating the effects of ER stress on cell death.

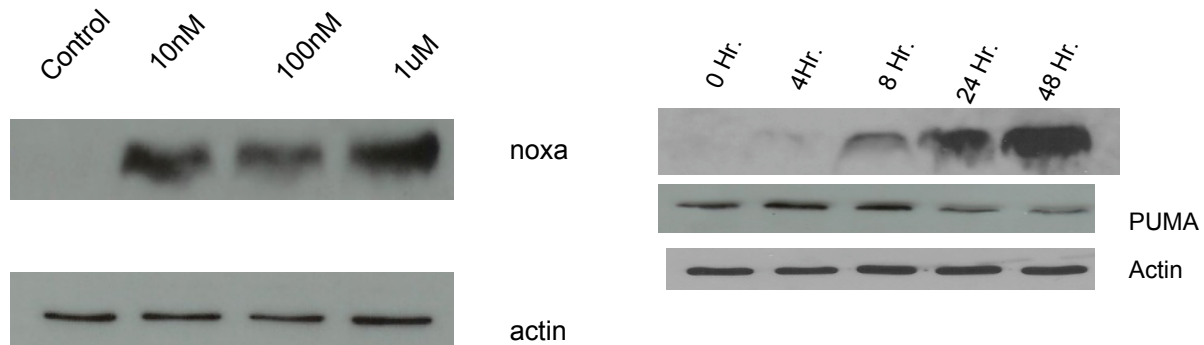


Figure 5: Effects of bortezomib on expression of Noxa and Puma. Left panels: Dose-response. LNCaP-Pro5 cells were incubated with the indicated concentrations of bortezomib for 24 h and Noxa expression was measured by immunoblotting. Right panels: Time course. LNCaP-Pro5 cells were incubated with 10 nM bortezomib for the times indicated and expression of Noxa and Puma was measured by immunoblotting. Note rapid accumulation of Noxa (top panel), easily detectable by 8 h. Also note that Puma expression declines at 24-48 h. This decline is not blocked by caspase inhibitors, indicating that it is not caused by apoptotic cell death.

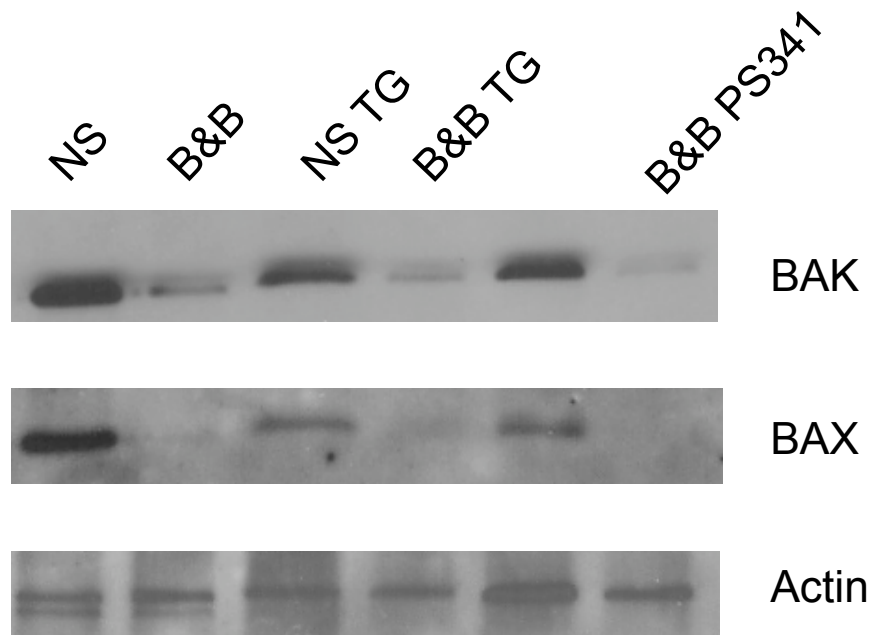


Figure 6: Efficiency of Bax and Bak silencing. LNCaP-Pro5 cells were transfected with siRNAs targeting Bax and Bak (B&B) for 48 h. Cells were then exposed to thapsigargin for 48 h and expression of the proteins was measured by immunoblotting. Similar results were obtained in cells transduced with one or the other construct.

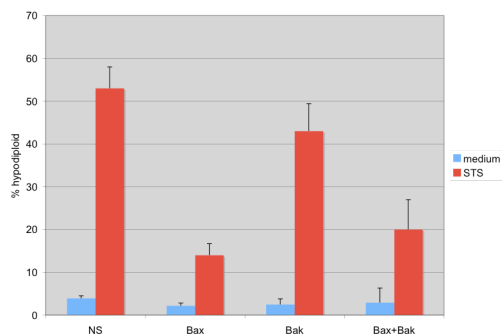


Figure 7: Effects of Bax or Bak silencing on apoptosis induced by staurosporine. Cell death was quantified by PI/FACS. Mean \pm SD, n = 3.

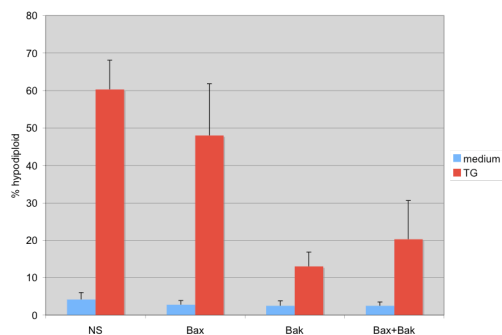


Figure 8: Effects of Bax or Bak silencing on apoptosis induced by thapsigargin. Cell death was quantified by PI/FACS. Mean \pm SD, n = 3.

Figure 9: Effects of Bax or Bak silencing on apoptosis induced by bortezomib. Cell death was quantified by PI/FACS. Mean \pm SD, n = 3.

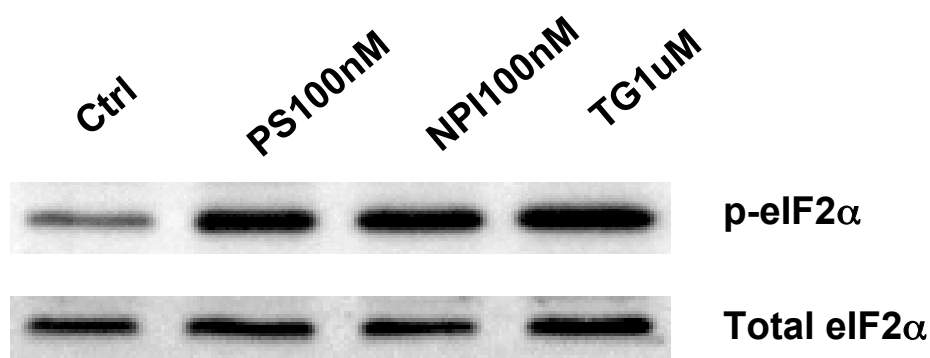
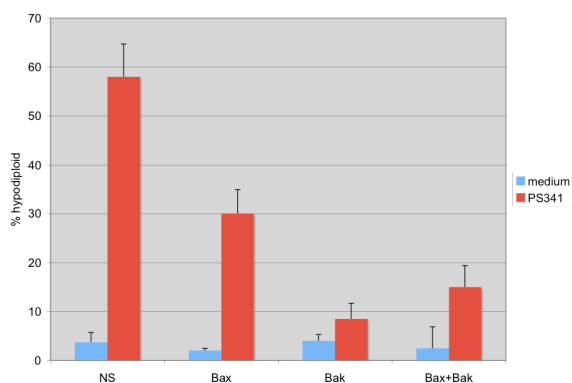


Figure 10: Effects of proteasome inhibitors or thapsigargin on phosphorylation of the translation initiation factor eIF2 α . LNCaP-Pro5 cells were incubated with bortezomib (PS), NPI-0052 (NPI), or thapsigargin (TG) for 4 h and phosphorylated and total eIF2 α were measured by immunoblotting. Identical results were obtained with PC-3 cells.

Summary: Initial studies performed in the first year of funding confirmed that wild-type and ER-targeted forms of BCL-2 blocked ER-to-mitochondrial Ca^{2+} fluxes and apoptosis in prostate cancer cells, but concerns about the stability of protein expression and the physiological relevance of overexpression systems in general prompted us to adopt an alternative strategy. By using transient siRNA to knock down BH3-only and multi-domain proapoptotic members of the BCL-2 family we were able to generate more definitive conclusions about their contributions to cell death induced by our panel of proapoptotic stimuli. We confirmed our prediction that staurosporine-induced apoptosis is dependent on the BH3-only protein, Bim and the multidomain family member, Bax. In ongoing studies we will use this system to study the effects of staurosporine on intracellular Ca^{2+} fluxes to determine whether or not Bim and Bax act upstream or downstream of ER Ca^{2+} release (study #1). We will also study the direct effects of Bim and Bax on ER Ca^{2+} release in isolated microsomes as described in Aim 1 of the proposal. We will use a new source of institutional funding to “leverage” the funds we have received from the PCRP and plan to complete this project within the next year. Our results are consistent with recent work by other investigators (28).

The project also spawned a new, second project that is closely related to the first. Thapsigargin is an ER Ca^{2+} ATPase inhibitor that bypasses the biochemical mechanisms elicited by staurosporine and certain other pro-apoptotic stimuli to directly release the ER Ca^{2+} pool. Apoptosis induced by thapsigargin, and to a lesser extent by two clinically relevant inhibitors of the proteasome (NPI-0052 and bortezomib), was highly dependent upon expression of the multi-domain pro-apoptotic protein, Bak, and our data demonstrate that ER stress mediates the cell death that is induced by both agents. When Bak was first isolated it was thought to localize to the ER (Robin Brown, personal communication), although now most investigators think of it as constitutively localized to the outer mitochondrial membrane. We plan to more carefully study the subcellular localization and function of Bak in prostate cancer cells. The work also initiated a new project that focuses on the role of autophagy in limiting proteasome inhibitor-induced ER stress in prostate cancer cells (manuscript in preparation, proposal for funding submitted), which has important therapeutic implications given that an autophagy inhibitor (the anti-malarial drug chloroquine) is already approved for use in humans.

KEY RESEARCH ACCOMPLISHMENTS

- Showed that Bax and Bak play distinct roles in ER stress-induced apoptosis
- Obtained evidence that proteasome inhibitors and thapsigargin kill prostate cancer cells via ER stress (involving eIF2 α phosphorylation, GADD153/CHOP accumulation, and caspase-4 activation)
- Implicated the BH3-only protein Noxa in proteasome inhibitor-induced cell death

REPORTABLE OUTCOMES

- Manuscript describing the effects of thapsigargin and proteasome inhibitors on ER stress is being finalized for submission
- Manuscript describing the effects of proteasome inhibitors on autophagy is in preparation

CONCLUSIONS

- ER-targeted (but not mitochondrial) BCL-2 blocks ER-to-mitochondrial Ca^{2+} fluxes
- Enforced expression of wild-type and particularly organelle-targeted (mitochondrial) forms of BCL-2 is unstable in human prostate cancer cells.
- Bim and Bax appear to play central roles in staurosporine-induced cell death
- Noxa and Bak appear to play central roles in ER stress-induced cell death

- ER stress mediates the cell death that is induced by proteasome inhibitors or thapsigargin

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